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SUPPLEMENTAL MATERIALS AND METHODS

Study population

A total of 14 patients with asthma, 17 COPD patients, and 19 normal controls were prospectively recruited from the Centre Hospitalier Universitaire of Bordeaux.

Asthmatic patients

Asthmatic patients were enrolled using the following inclusion criteria. Patients >18 yr had to exhibit characteristic symptoms (i.e., wheezing and breathlessness), as well as bronchial hyperresponsiveness confirmed either by a significant improvement by >15% in the forced expiratory volume in 1 s (FEV_1) 10 min after the inhalation of 200 μg of salbutamol, or a provocative concentration of methacholine required to lower the FEV_1 by 20% (PC20) of <4 mg/ml according to the American Thoracic Society criteria (1). Patients must all be graded as severe persistent asthmatics according to the Global Initiative for Asthma guidelines based on both clinical features and daily medication regimen (2). All patients must also be lifelong nonsmokers with no recent exacerbation (<3 mo). Exclusion criteria were recent bacterial or viral infections (<3 mo).

COPD patients

COPD patients were enrolled using the following inclusion criteria. Patients >18 yr had to present chronic symptoms (i.e., cough and breathlessness) and poorly reversible airflow obstruction defined by both an improvement of <10% in the FEV_1 10 min after the inhalation of 200 μg of salbutamol or ipratropium bromide, and a postbronchodilator FEV_1/forced vital capacity ratio <70% according to the Global Initiative for Chronic Obstructive Lung Disease (GOLD) guidelines (3). Patients must be graded as moderate to severe COPD according to the GOLD guidelines (3). Exclusion criteria were recent bacterial or viral infections (<3 mo).

Control subjects

Control subjects were enrolled using the following inclusion criteria. Subjects >18 yr had to have normal lung function test and have underwent a fiberoptic fibroscopy or a thoracotomy because of hemoptysis or abnormal image on x ray. They must be asymptomatic without any treatment, and only those in whom fiberoptic investigation and bronchial mucosa were normal were selected as controls. Exclusion criteria were any comorbidity, such as asthma or COPD, and recent bacterial or viral infections (<3 mo).

All subjects gave their written informed consent to participate in the study after the nature of the procedure had been fully explained. The study followed recommendations outlined in the Helsinki Declaration and received approval from the local ethics committee.

Study procedures

Bronchial specimens.

Bronchial specimens were obtained by either fiberoptic bronchoscopy or lobectomy, as previously described (4, 5). Fiberoptic bronchoscopy was performed after the anesthesia of the upper airways was achieved with lidocaine 10% spray. The fiberoptic bronchoscope (FB-5V; Pentax) was introduced, and biopsies were taken from various bronchial carinae from the middle lobe.

Because the smooth muscle remodeling in both asthma and COPD occurred at two different bronchial localizations, we thus collected asthmatic bronchial specimens from the third to the fourth generation, those from COPD patients from the fourth to the sixth generation and those from control subjects were collected from the third to the sixth generation. Specimens were immediately transferred to the laboratory in a sterile container containing DME.

Optic microscopy and immunohistochemistry.

Bronchial specimens were fixed in 10% buffered formalin and processed in standard fashion for paraffin embedding. 3-μm-thick sections were cut and stained with hematoxylin-eosin-safran or processed for immunohistochemistry. After deparaffinization and rehydratation through graded alcohols, endogenous peroxidase was inhibited using a solution of 0.1% sodium azide and 0.3% hydrogen peroxide for 30 min, followed by two rinses in PBS (Invitrogen). Nonspecific staining was blocked using 10% BSA (Dako) for 30 min. Sections were incubated for 2 h at room temperature with mouse anti–human smooth muscle actin (clone 1A4) or an unrelated mouse antibody (both from Sigma-Aldrich). After rinsing in PBS, biotinylated rabbit anti–mouse F(ab')2 (Dako) was applied to the sections for 2 h, followed by the streptavidin-biotinylated horseradish peroxidase complex (Dako) for an additional 2 h. After rinsing in PBS, 1 mg/ml 3,3′-diaminobenzidine tetrahydrochloride (Dako), plus 0.02% hy-

drogen peroxide for 6 min, were used as substrate to develop a peroxide-dependent brown color reaction at room temperature. Finally, the sections were rinsed and counterstained with Mayer's hematoxylin. There was assessable BSM in the bronchial specimens from all 14 asthmatics, 17 COPD patients, and 19 controls. The total area of smooth muscle layer was assessed manually in a blinded fashion using ScanView software (Soft Imaging System) at a magnification of 200×. This smooth muscle area was normalized by the whole area of the corresponding tissues and presented as percentages of whole area.

Electron microscopy.
Specimens were fixed in 2.5% glutaraldehyde in cacodylate buffer, postfixed in 1% osmium tetroxide, dehydrated, and embedded in epon. For each specimen, semithin sections (1 μm thick) were cut and stained with alkaline toluidine blue. We selected the first semithin section large enough to span from the epithelium to the muscular layer. 10 ultrathin serial sections (60 nm thick) were then cut on diamond knives. Three of these latter sections were subsequently randomly selected and placed on grids. Staining was performed with uracile acetate and lead citrate. Grids were then scanned by transmission electron microscopy (Tecnai 12; Philips) and examined by a pathologist from left to right and from top to bottom to locate every whole nucleated BSM, epithelial, and endothelial cell. Each ultrathin section was examined in its entirety. To evaluate the number of mitochondria, computerized photographs and measurements were performed in a blinded fashion by using ScanView software at a standard magnification of 6,000×. Mitochondrial densities were performed using ImageJ 1.34s free software (National Institutes of Health).

Cell cultures.
Primary cultures of BSM cells were established from bronchial specimens. After a fine dissection under a microscope, smooth muscle explants were cultured in six-well plates in a humidified atmosphere at 37°C with 5% CO_2. Cells were maintained in sterile DME containing 10% (vol/vol) FCS and 4.5 g/liter glucose, supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericine B (antimycotic/antibiotic solution; all from Invitrogen), 1 mM sodium pyruvate, and 1% (vol/vol) nonessential amino acid mixture (both from Sigma-Aldrich). The medium was changed every 48–72 h. After 6–8 wk, confluent cells were rinsed twice with HBSS and passaged with trypsin-EDTA (both from Invitrogen).

To assess purity of the cells, an immunocytochemical method was used, using an indirect immunofluorescence technique. Cells of varying passage number were growth arrested by incubating the cells with serum-free DME supplemented with 10 μg/ml insulin, 5.5 μg/ml transferrin, 5 ng/ml selenium, 0.5 μg/ml BSA, and 4.7 μg/ml linoleic and oleic acid (ITS solution; Sigma-Aldrich). After 48 h, cells were rinsed twice in PBS and fixed with cold methanol for 20 min. Nonspecific staining was blocked using PBS containing 3% BSA for 30 min. Monoclonal antibodies (mAb) diluted in PBS with 1% BSA, including anti–α-smooth muscle actin (Sigma-Aldrich), anti–smooth muscle myosin (Sigma-Aldrich), anti-cytokeratin 18 (Sigma-Aldrich), and anti-factor VIII (Dako) were incubated for 1 h. Control slides were treated similarly, using an unrelated antibody (mouse IgG; Sigma-Aldrich). After rinsing with PBS containing 0.05% Tween20 (Sigma-Aldrich), the cells were incubated for 1 h with FITC-conjugated anti–mouse immunoglobulins (Dako). Slides were mounted with a drop of fluorescent mounting medium (Dako) and observed under a fluorescence microscope (Nikon). Depending on the experiments, cells with a confirmed smooth muscle phenotype were seeded on coverslips, chamber slides, 75-cm^2 culture flasks, and 6-, 12-, or 96-well plates. Only cells at passage 2 to 4 were used for this study.

To analyze the mitochondrial ultrastructure, BSM cells were growth arrested, as described in the previous section, for 6 d. 3×10^6 cells were then collected and centrifuged at 400 *g* for 5 min. Cell pellets were fixed in 2.5% glutaraldehyde for 2 h at 4°C and processed for electron microscopy, as described in the previous section. Growth curves were built to assess the role of mitochondria in cell proliferation. For this purpose, 25,000 BSM cells were plated on 75-cm^2 flasks and cultured with 10% FCS-DME containing either 4.5 g/liter galactose or 4.5 g/liter glucose. In another set of experiments, we investigated the effects of 50 μg/liter ethidium bromide (Qbiogene) for up to 33 d, 1.3 g/liter cyclic cGMP (3 mM; Sigma-Aldrich) for up to 6 d, or 0.5 g/liter D600, i.e., methoxyverapamil (1 μM; Sigma-Aldrich) for up to 2 d in 10% FCS-DME containing 4.5 g/liter glucose. For these latter experiments, medium was changed every 24 h, and, at the indicated time, BSM cells were harvested by trypsinization and counted in triplicate using a cytometer.

Immunoblotting.
Whole lysates from BSM cells were collected using 1% Triton X-100 lysis buffer in the presence of 2 mM Na orthovanadate, 1 mM EDTA, 50 μg/ml aprotinin, 100 μM leupeptin, 1 mM 1.4 Dithio-DL-Treitol, and 1 mM amino-ethyl-benzenesulfonyl fluoride hydrochloride (all from Sigma-Aldrich). Cellular extracts were reduced with mercaptoethanol, subjected to electrophoresis on a 10% acrylamide reducing gel, and transferred to PVDF membranes (Immobilon TM-P; Millipore). The immunoblots were then developed using mouse anti–human porin (MitoSciences), mouse anti–human β-actin (Sigma-Aldrich), rabbit anti–human phospho-CaMK-IV, rabbit anti–human PGC-1α, rabbit anti–human NRF-1, or rabbit anti–human mitochondrial transcription factor A (mtTFA; all purchased from Santa Cruz Biotechnology, Inc.) for 14 h at 4°C. For amplifica-

tion, biotinylated goat secondary antibody anti–mouse IgG (Bio-Rad Laboratories) or anti–rabbit IgG (Santa Cruz Biotechnology, Inc.) for 2 h at room temperature and a streptavidin-biotinylated horseradish peroxidase complex (Dako) were used. Immunoblots were revealed by enhanced chemiluminescence (Uptima). Blot images were acquired using BioCaptMW (Thermo Fisher Scientific), and band densities were quantified using ImageJ software.

Endogenous cell oxygen consumption and determination of the coupling ratio.
Endogenous cellular oxygen consumption was monitored on intact cells at 37°C in a 1-ml thermostatically controlled chamber (10^6 cells/ml/run) equipped with a Clark oxygen electrode (Oxygraph System; Hansatech). The respiratory buffer was the glucose or the galactose culture medium described in Cell culture. The endogenous respiratory rates were expressed in ngatom O/min/10^6 cells. To assess the mitochondrial coupling, respiratory rates were monitored using inhibitors of the phosphorylation system, i.e., 1 mM atractyloside, 1 mM carboxyatractyloside, and 1 nM oligomycin in combination. Such inhibitors are considered as decouplers and can be used to evaluate the degree of mitochondrial ATP synthesis (6). Control experiments were performed using 1 mM cyanide as a blocker of the respiratory chain.

Three-dimensional assessment of the mitochondrial network organization.
To visualize the mitochondrial network, we targeted a GFP to the mitochondrial matrix space of the BSM cells. For this purpose, we used the mitochondrion-targeted GFP plasmid (mito-GFP), which was derived from the plasmid pEGFP-N1 (Clontech Laboratories, Inc.) containing the leader sequence of the E1α subunit of pyruvate dehydrogenase. 1 d before transfection, BSM cells were plated at 50% confluency in 10% FCS-DME without antibiotics. Transfections were conducted for 6 h at 37°C in 5% CO_2 with 1.5 μg of plasmid and 1.7% of the cationic lipid Lipofectamine 2000 (Invitrogen) in the absence of FCS. After 72 h, BSM cells were rinsed in PBS and fixed with 4% paraformaldehyde (PFA) for 20 min on ice. After drying, the slides were mounted with fluorescent mounting medium (Dako). Confocal differential interference contrast images were obtained using the FluoView laser scanning microscope (Nikon) and 60× oil-immersion objective. Z series sections were recorded in successive z axis serial sections at 0.5-μm intervals, and they were composed of optical sections in the x-y optical plane. Sections were reconstituted in three-dimensional images using Imaris Software (Bitplane).

RNA extraction, RT, and real-time quantitative PCR.
BSM cells were washed twice with HBSS and lysed by the addition of 300 liters/well Trizol (Invitrogen) and 30 μl of chloroform (Sigma-Aldrich). The RNA was extracted from the aqueous phase after being centrifuged at 12,000 *g* for 15 min. RNA was precipitated in the presence of isopropanol (Sigma-Aldrich) at −20°C overnight. The pure RNA was recovered by centrifugation and washed with 80% ethanol (Sigma-Aldrich). The purity was assessed by electrophoresis (30 mA for 75 min) on 1.5% agarose gel (Bio-Rad Laboratories), followed by 20 min incubation in 10 μg/ml ethidium bromide (Sigma-Aldrich), showing 2 bands corresponding to the 28S and 18S fractions of ribosomal RNA. The concentration of RNA was measured spectrophotometrically by GeneQuant RNA/DNA calculator (GE Healthcare). The total RNA (1 μg) was reverse transcribed into cDNA using AMV RT (Promega), RNase inhibitor, and oligo d(T) as a primer at 42°C for 60 min, followed by heating at 94°C for 3 min.

Real-time quantitative PCR was performed on a Rotor-Gene 2000 (Corbett Research). In brief, triplicate PCR reactions were assembled in 0.1-ml strip tubes containing cDNA from 10 ng of total RNA, 0.2 μl 50× Titanium Taq DNA Polymerase, 1× Titanium Taq PCR Buffer (Clontech Laboratories, Inc.), 1 mM dNTP, 100 mM each of the appropriate primer, and 0.5× Sybr Green I (Invitrogen). The PCR was performed under the following conditions: denaturation at 95°C for 15 s, annealing temperature (64–72°C) depending on specific primers for 15 s, and extension at 70°C for 30 s for 30–40 cycles. Data collection was performed after each extension step, at a temperature at least 3.5°C lower than the melting temperature of the amplicon (generally between 80–85°C) to eliminate nonspecific fluorescence signal. PCR-negative controls were systematically made using water instead of cDNA or RNA sample without the RT step. All specific primers were designed using the primer analysis software (Oligo 6.6; Molecular Biology Insights) and were ordered from Sigma-Aldrich. Primers, sense and antisense, were as follows: for mtTFA (NM_003201), forward 5′-GAAGTCGACTGCGCTCCC-3′ and reverse 5′-ACTCCGCCCTATAAGCATC-3′; for PGC-1α (NM_013261), forward 5′-GTCACCACCCAAATCCTTATT-3′ and reverse 5′-GGCGATCTTGAACGTGAT-3′; for NRF-1 (NM_005011), forward 5′-AAGATCAGCAAACGCAAACAC-3′ and reverse 5′-CCCGTACCAACCTGGATAAGT-3′; for tyrosin 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide (YWHAZ) (NM_003406), forward 5′-GGGGATGTGGAATTTTTATACAAG-3′ and reverse 5′-CGCCAGGACAAACCAGTATGTAG-3′; for hypoxanthine phosphoribosyltransferase 1 (HPRT-1) (NM_000194), forward 5′-GTGAAAAGGACCCCACGAA-3′ and reverse 5′-AGTCAAGGGCATATCCTACAACAA-3′; and for human acidic ribosomal phosphoproteins (PO) (NM_001002), forward 5′-CAACGGGTACAAACGAGTC-3′ and reverse 5′-CTTCCTTGGCTTCAACCTTAG-3′. The efficiency of the PCR reactions was always >90%. The specificity of the amplified PCR products was examined with the melting curve analysis, and also in 2% agarose gel containing ethidium bromide. The RT-PCR expression of the target genes (i.e., mtTFA, PGC-1α, and NRF-1) was thus presented as an arbitrary unit and

normalized to endogenous references (geometric averaging of three internal control genes; i.e., YWHAZ, HPRT-1, and PO) according to geNorm software (7).

Microspectrofluorimetry.
Change in growth-arrested BSM cell intracellular calcium concentration ($[Ca^{2+}]_i$), was assessed using the Ca^{2+}-sensitive probe indo-1, as previously described (8, 9). In brief, cells were loaded with indo-1 (Calbiochem) and mounted in a perfusion chamber continuously perfused with phosphate salt solution containing either 2 mM Ca^{2+} in the absence or presence of 1 μM D600, or 400 μM EGTA (Sigma-Aldrich) in the absence of extracellular calcium. BSM cells were stimulated with 10^{-5} M acetylcholine or histamine (both from Sigma-Aldrich). Individual cell calcium levels were monitored continuously. Results were expressed as the mean ± the SEM of resting or peak of $[Ca^{2+}]_i$ rises (in millimoles) and area under the curves (nanomoles/second). Experiments were carried out at room temperature (22–25°C).

BrdU incorporation.
Growth-arrested BSM cells were cultured in 96-well plates for 24 h and stimulated with 10% FCS-DME containing glucose, galactose, ethidium bromide, or cyclic GMP for 24 h or containing D600 for 48 h, as described above. Control BSM cells remained in serum-deprived DME. DNA synthesis was measured using the BrdU kit according to the manufacturer's instructions (Roche). In brief, BSM cells were incubated with 10 μM BrdU for 2 h at 37°C and fixed for 20 min at room temperature. Cells were denaturized for 30 min and incubated with the anti-BrdU antibody for 2 h at room temperature. Absorbance was measured at 370 nm in a microplate reader in triplicate. Results are expressed as a normalized ratio of BrdU incorporation, i.e., absorbance for test condition divided by absorbance for control serum-deprived condition.

Statistical analysis.
The statistical analysis was performed with NCSS 2001 software. Comparisons between the three groups were performed by means of ANOVA, with the use of Bonferroni's test for multiple comparisons or paired Student's *t* tests. Values are presented as the mean ± the SEM. A Pearson correlation matrix was built between in vitro and in vivo measurements. A P value <0.05 was considered statistically significant.

REFERENCES

1. Crapo, R.O., R. Casaburi, A.L. Coates, P.L. Enright, J.L. Hankinson, C.G. Irvin, N.R. MacIntyre, R.T. McKay, J.S. Wanger, S.D. Anderson, et al. 2000. Guidelines for methacholine and exercise challenge testing-1999. This official statement of the American Thoracic Society was adopted by the ATS Board of Directors, July 1999. *Am. J. Respir. Crit. Care Med.* 161:309–329.
2. Global Initiative for Asthma. 1995. Global strategy for asthma management and prevention. NIH publication (updated 2005). http://www.ginasthma.com/download.asp?/intId=87.
3. Global Initiative for Chronic Obstructive Lung Disease. 1998. Global strategy for the diagnosis, management and prevention of chronic obstructive pulmonary disease. NIH publication (updated 2005). http://www.goldcopd.com/download.asp?intId=231.
4. Berger, P., P.O. Girodet, H. Begueret, O. Ousova, D.W. Perng, R. Marthan, A.F. Walls, and J.M. Tunon De Lara. 2003. Tryptase-stimulated human airway smooth muscle cells induce cytokine synthesis and mast cell chemotaxis. *FASEB J.* 17:2139–2141.
5. El-Shazly, A., P. Berger, P.O. Girodet, O. Ousova, M. Fayon, J.M. Vernejoux, R. Marthan, and J.M. Tunon-de-Lara. 2006. Fraktalkine produced by airway smooth muscle cells contributes to mast cell recruitment in asthma. *J. Immunol.* 176:1860–1868.
6. Benard, G., N. Bellance, D. James, P. Parrone, H. Fernandez, T. Letellier, and R. Rossignol. 2007. Mitochondrial bioenergetics and structural network organization. *J. Cell Sci.* 120:838–848.doi:10.1242/jcs.03381
7. Vandesompele, J., K. De Preter, F. Pattyn, B. Poppe, N. Van Roy, A. De Paepe, and F. Speleman. 2002. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol.* 3. doi:10.1186/gb-2002-3-7-research0034
8. Berger, P., J.M. Tunon-de-Lara, J.P. Savineau, and R. Marthan. 2001. Tryptase-induced PAR-2-mediated Ca(2+) signaling in human airway smooth muscle cells. *J. Appl. Physiol.* 91:995–1003.
9. Trian, T., P.O. Girodet, O. Ousova, R. Marthan, J.M. Tunon-de-Lara, and P. Berger. 2006. RNA interference decreases PAR-2 expression and function in human airway smooth muscle cells. *Am. J. Respir. Cell Mol. Biol.* 34:49–55. doi:10.1165/rcmb.2005-0187OC